A VOLATILE FACTOR IN RELATION TO IN VITRO SPERMATOGENESIS IN THE CECROPIA SILKWORM[1]

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In one of the few successful applications of tissue culture techniques to insects, Schmidt and Williams (1953) were able to culture the spermatogonia and spermatocytes of diapausing silkworm pupae in simple hanging-drops of the insect's blood. The same technique in slightly modified form was subsequently used by Schneiderman, Ketchel and Williams (1953) in a study of the metabolism of spermatogenesis. These investigations, in brief, showed that dormant cysts of the male sex cells develop promptly into bundles of spermatids and spermatozoa when cultured in blood obtained from non-diapausing donors. Since comparable development failed to occur in cultures prepared in the blood of diapausing pupae, such blood was presumed to be deficient in a growth factor present in the blood during the non-diapausing stages. Indeed, it proved feasible to use the *in vitro* technique as a method of assay for this growth factor. The latter, on the basis of several lines of evidence, was considered to be the prothoracic gland hormone—the primary stimulus for cellular growth and metamorphosis within the insect as a whole.

In the course of the studies just considered, it was observed that cultures prepared in individual depression slides failed to develop and, in fact, underwent more or less prompt degeneration unless closed off from communication with the outside air. Moreover, in experiments utilizing larger culture chambers, it proved imperative, not only to seal the chambers tightly, but also to place a number of cultures in each chamber. Thus, an individual culture when isolated in a large sealed chamber under otherwise optimal conditions underwent far less development than when a number of other cultures were simultaneously present in the same chamber. This latter practice of placing numerous cultures in each chamber was routinely adopted by Schneiderman, Ketchel and Williams (1953) as an empirical solution of the problem. However, it was clear that the phenomenon was of sufficient interest in itself to merit further study. Consequently, we have returned to the initial problem in an effort to define the basis for the interaction of cultures in the same chamber.

MATERIALS AND METHODS

The experiments were performed on spermatocytal cysts obtained from diapausing pupae of the Cecropia silkworm according to the methods described by

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Schneiderman, Ketchel and Williams (1953). Diapausing pupae served as donors of "inactive blood"; "active blood" was obtained from post-diapausing individuals on the first day of adult development. In order to block the tyrosinase reaction, all samples of blood were saturated with phenylthiourea.

Each culture chamber consisted of a brass ring (4″ in inside diameter and 0.08″ in thickness) sandwiched between a pair of glass plates (5″ × 5″ × 0.06″) and sealed together with melted paraffin. The plates had previously been given a hydrophobic coating with silicone ("Dryfilm 9987," General Electric Company). The chamber enclosed a gas volume of 17 cc. and could accommodate up to twenty sitting-drop cultures of approximately 20 $mm.^3$ each. In experiments utilizing gas mixtures, the brass ring was equipped with stainless steel inlet and outlet tubes as previously described.

In all save a few special cases a minimum of two such chambers were utilized in each experiment; namely, a control chamber containing 16 cultures in active blood, and an experimental chamber containing one or more cultures prepared from the same blood and cyst suspension. After seven days at 25° C., 30 to 40 per cent of the cysts in the control cultures had ordinarily progressed to Stage III (spermatidal cysts) or IV (spermatozoal cysts). The experiment was terminated at this point and the degree of development in each chamber ascertained. For this purpose the number of cysts which had developed to Stages III or IV was recorded by counting a total of 500 cysts in a series of randomly selected low-power fields. Ordinarily the response in the control cultures was taken as 100 per cent, and the response in the experimental cultures calculated as a percentage of that of the controls.

INFLUENCE OF THE NUMBER OF CULTURES PER CHAMBER

The quantitative effects of the number of cultures enclosed in each sealed chamber were studied in detail in two series of experiments. In the first experiment a suspension of cysts was prepared in active blood and distributed among five chambers so that the number of cultures per chamber ranged from 0.5 to 8. Seven days later, counts were made of 500 cysts in each chamber and the percentage of the 500 in Stages III or IV recorded.

The summary of results in Table I shows that chambers containing fewer than 4 cultures supported the development of a small proportion of cysts. The response was enhanced in the 4-culture chamber and further enhanced in the 8-culture chamber.

TABLE I

The number of cultures in each sealed chamber conditions the developmental response

Cultures per chamber	Per cent response* Expt. 1	Expt. 2
16	—	45
8	21	43
4	14	12
2	8	15
1	2	13
½	4	—

* Per cent of the total cysts counted which had reached Stage III (spermatidal cysts) and Stage IV (spermatozoal cysts) after 7 days of culture.

The second experiment (Table I) utilized a cyst suspension which showed a greater degree of response in all preparations. Minimal development was found in the 1, 2, and 4-culture chambers. By contrast, the 8- and 16-culture chambers showed a three-fold increase in the proportion of cysts attaining Stages III or IV.

On the basis of numerous other experiments comparing the development attained in 1-culture and 16-culture chambers of the volume described above, we can state that the difference was never less than three-fold and commonly much greater than three-fold.

Absence of Stimulation by Non-Growing Cultures

The experiments just considered indicate that the development of the cysts in any one culture is promoted by the simultaneous presence of other cultures in the same sealed chamber. It is therefore worth inquiring whether this same influence can be exerted by non-growing cultures prepared in inactive blood, or, indeed, by drops of active or inactive blood in the total absence of cysts.

To test these possibilities, 16 cultures of a cyst suspension in active blood were placed in a control chamber, and one culture of the same suspension in each of four experimental chambers. Three of the experimental chambers then received, respectively, 15 drops of a cyst suspension in inactive blood, or 15 drops of active blood without cysts, or 15 drops of inactive blood without cysts.

The results computed as per cent of the response of the control cultures are recorded in Figure 1. It is obvious that none of these maneuvers served to increase the low level of response of the individual test cultures. Consequently, it is clear that the agency by which one culture interacts with another is generated only by cultures capable of growth; *i.e.,* by cultures containing both cysts and active blood.

Hormonal Requirement

Since the development of spermatocytes was already known to require the presence of the hormonal component of active blood, it seemed possible that the developmental factor derived from actively growing cultures might correspond to some volatile derivative of this same hormone. Under this circumstance a culture prepared in inactive blood should develop if enclosed in the same chamber with numerous cultures in active blood.

An experiment of this type was performed, one culture in inactive blood being placed in the same culture chamber with 16 cultures prepared in active blood. The results of this experiment were clear-cut and decisive: the cultures in active blood developed promptly, whereas no development occurred in the test culture in inactive blood. This finding demonstrates that the stimulating factor produced by developing spermatocytes cannot substitute for the hormone, and that the presence of the hormone is prerequisite for the response of spermatocytes to the stimulating factor.

Effects of Carbon Dioxide and Oxygen Tensions

It will be recalled, as mentioned in the introduction, that a single culture in the limited volume of a depression slide develops normally provided that the hormone is present and the cover-slip tightly sealed. But if the cover-slip is placed slightly ajar, then no development occurs and the cysts promptly die. This fact, in itself, directs attention to the gaseous medium within the sealed chamber and its critical modification by the developing culture contained therein. Evidently, in the case of the larger chambers utilized in the present study, a considerable number of cultures are required to "condition" the greatly enhanced gas volume.

The problem at this point seemed extremely simple since a basis for conditioning the gaseous medium was obviously present in the respiration of the cysts themselves.

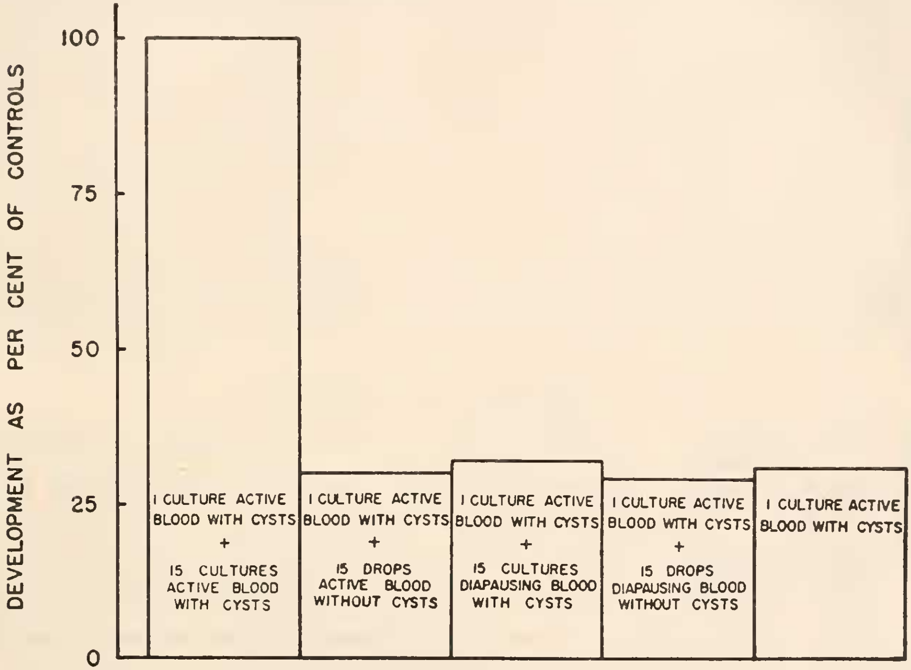

FIGURE 1. Interaction between individual cultures in the same chamber. The growth response of the test culture is enhanced by the presence of cultures containing spermatocytes in active blood, but is not enhanced if either of these components is absent.

In order to test whether the stimulating factor might correspond to a critical tension of carbon dioxide, two cultures of cysts in active blood were placed in one chamber and 16 cultures in each of two other chambers. One of the latter chambers then received, in addition, 16 drops of 0.1 *M* potassium hydroxide for the purpose of absorbing carbon dioxide. Seven days later the 2-culture control chamber showed the expected low percentage of development, while the 16-culture control chamber showed the anticipated high percentage. Of present interest and importance is the fact that the same high percentage of development was observed in the chamber containing the 16 cultures plus alkali. This experiment was repeated with the same uniform result. Consequently, a critical tension of carbon dioxide cannot be the stimulating factor.

The possibility that a critically low oxygen tension was the source of stimulation was a far less attractive hypothesis, since Schneiderman, Ketchel and Williams (1953) had already demonstrated that the *in vitro* development of spermatocytes was independent of oxygen tensions ranging from 1 to 21 per cent of one atmosphere. This finding was re-examined in the following experiment.

A series of mixtures of oxygen and nitrogen were prepared containing 20, 15, 10, 5, and 0.5 per cent oxygen, respectively. Two cultures of cysts in active blood were placed in each of five experimental chambers and 16 cultures in a sixth chamber that served as a control. Each experimental chamber was flushed for five minutes daily with one of the above-mentioned gas mixtures in order to establish and maintain a particular oxygen tension. Each experiment was performed in duplicate.

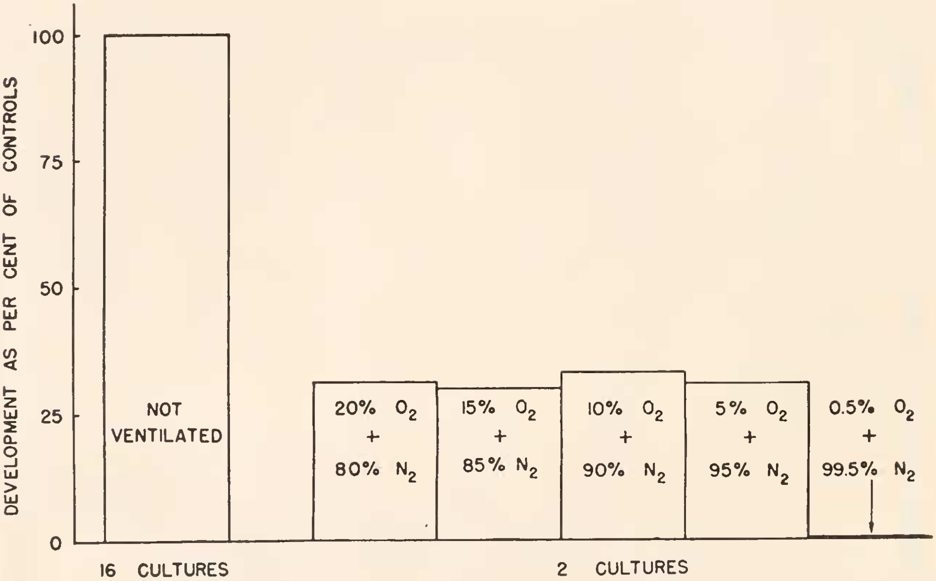

FIGURE 2. Daily ventilation with gas mixtures containing specific low oxygen tensions fails to enhance the developmental response of the two cultures in each of a series of five chambers. The experiment was performed in duplicate series.

The results recorded in Figure 2 show that the developmental response was independent of oxygen at tensions ranging from 5 to 20 per cent, whereas in 0.5 per cent oxygen development was completely blocked. Consequently, the stimulating factor can scarcely correspond to a critical oxygen tension established by respiration.

CONDITIONING OF THE CULTURE MEDIUM

Though unresolved at this point, the "conditioning" of the gaseous medium is reminiscent of the conditioning of the culture medium which, in the case of vertebrate tissue cultures, is thought to precede the growth response (Parker, 1950). As is now generally recognized, the cells of vertebrates must first react with the culture medium to establish a critical concentration of some unknown factor before growth becomes possible. Consequently, the establishment of a culture from a single vertebrate cell was successfully accomplished only after Sanford, Earle and Likely (1948) developed the technique of initiating such cultures in a capillary tube containing a minute volume of medium. In order to determine whether, in the case of spermatocytes, a conditioning of the culture medium precedes the conditioning of the gaseous medium, the following experiment was performed.

A cyst suspension was prepared in active blood and distributed as two cultures in each of four chambers. The residual cyst suspension was then diluted with seven volumes of the same sample of active blood; the diluted suspension was distributed as 16 cultures in each of four chambers. Consequently, all chambers contained the same total number of cysts (*ca.* 800), whereas the volume of culture medium differed by eight-fold in the two series. It was reasoned that if the same number of cysts developed in all chambers, then any influence of the conditioning of the medium could be disregarded. Alternatively, if development was enhanced in the more concentrated cultures, then the conditioning of the culture medium must be taken into account.

The answer provided by the experiment was neither of these possibilities, but yet a third; namely, an average of 36 per cent of the cysts developed in the more dilute suspensions whereas only 10 per cent developed in the concentrated ones. Consequently, under the conditions of the experiment, the presence of the larger volume of blood per cyst resulted in the greater response.

THE VOLATILE FACTOR

1. *Effects of ventilation*

A suspension of cysts in active blood was distributed among eight chambers, two cultures being placed in each of four chambers, and eight cultures in each of four. All eight chambers were immediately flushed for five minutes with a gas mixture containing 20 per cent oxygen and 80 per cent nitrogen. This same flushing procedure was repeated daily for two of the four chambers of each series, the other chambers remaining sealed as controls.

As recorded in Figure 3, the results were clear-cut in that development in the ventilated cultures was greatly depressed. Indeed, development in the ventilated 8-culture chambers was depressed virtually to the level observed in unventilated 2-culture chambers.

2. *Solubilities of the factor*

Eight cultures in active blood were placed in each of a series of chambers along with 8 to 10 drops of various solvents; namely, 0.16 M HCl, 0.16 M KOH, 0.1 M H_2SO_4, 0.32 M sucrose, light mineral oil, or inactive blood. None of these solvents was able to depress development when tested in this manner. Consequently, the experimental procedure was modified to increase the surface area of the various solvents. For this purpose each chamber was subdivided by a barrier consisting of a folded strip of filter paper, and the latter saturated with solvent. Sixteen cultures in active blood were placed on one side of the barrier and one culture on the other side. In this manner, any volatile agent generated on the "donor" side of

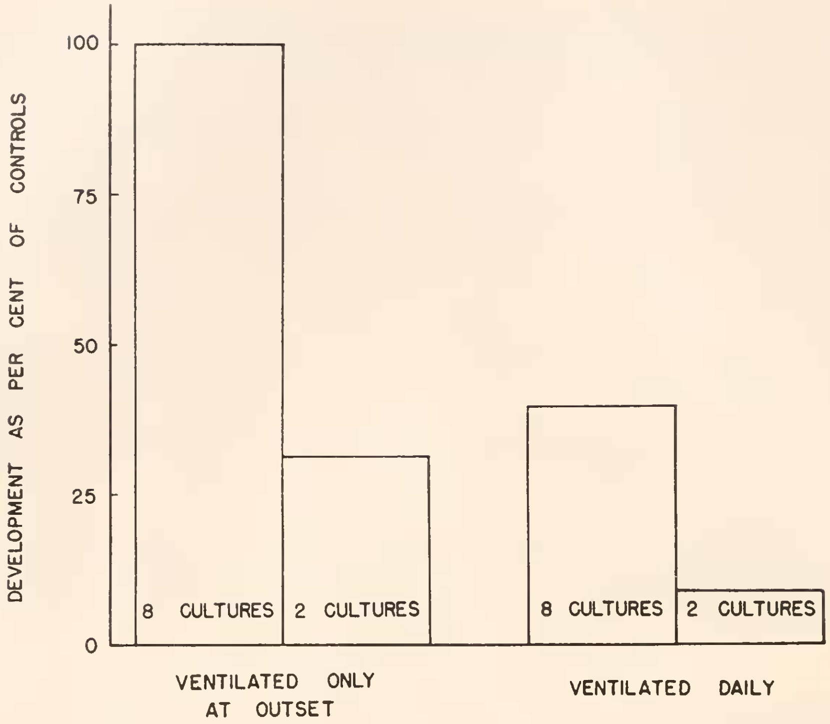

FIGURE 3. The daily ventilation of the chambers with air inhibits the developmental response. For further explanation, see text.

the chamber was forced to traverse the broad surface of solvent before reaching the recipient drop on the other side.

The results summarized in Table II show that acid, base, sugar solution, and Ringer's solution were ineffective when tested. However, mineral oil now produced a slight but definite inhibition. Of special interest and importance is the fact that inactive blood, spread upon the filter paper, considerably reduced the development on both sides of the chamber. Indeed, the development of the re-

TABLE II

The per cent of cysts developing in a single culture separated from fifteen other cultures by a barrier moistened with one of the solvents noted

Solvent	Per cent development
Insect Ringers solution	34
0.16 *M* NCl	37
0.16 *M* KOH	34
0.1 *M* H_2SO_4	32
0.32 *M* sucrose	35
Light mineral oil	24
Inactive blood	0 (cysts darkened and swollen)

cipient drop was totally suppressed and the cysts therein soon became dark, swollen, and obviously dead.

3. *Adsorption on activated charcoal*

As diagrammed in Figure 4, 16 cultures in active blood were distributed in three rows on one side of each of four chambers, and on the other side was placed 0.5 to 1 gm. of activated charcoal ("Norit A"). A fifth chamber lacking charcoal was used as a control. In the row of cultures nearest to the charcoal, development was completely suppressed: within twenty-four hours all the cysts in this row were swollen, dark, and dead. The cysts in the intermediate row of cultures appeared to remain viable, but only 2 per cent developed. In the row of drops furthest from the charcoal, the cysts remained viable and 14 per cent developed. In the control culture lacking charcoal, 42 per cent of the cysts developed.

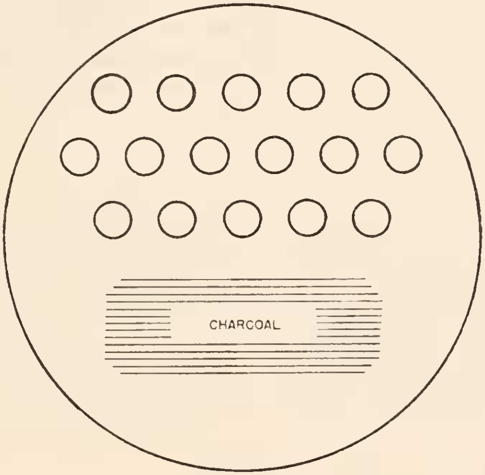

FIGURE 4. Semi-diagrammatic representation of the arrangement of cultures and charcoal in test chambers.

The experiment was repeated using a fresh cyst suspension and a second sample of Norit A from an unbroken package. The same results were obtained.

In order to eliminate the possibility that the charcoal released some toxic volatile principle, control chambers were ventilated with a current of air drawn through a column of charcoal and then sealed. Normal development was observed in all cases.

We therefore conclude that the volatile agent is adsorbed by activated charcoal. Evidently, in the above-mentioned experiments the adsorption was limited by the diffusion of the factor across the chamber to the charcoal from its site of production in the cultures. The most distant cultures preserved a sufficient local concentration of the agent to permit survival and a limited developmental response. In cultures closest to the charcoal, the concentration of factor was necessarily minimal.

It is of particular interest that such cultures not only failed to develop, but also underwent abnormal changes leading to death.

4. *Effects of charcoal on cultures in inactive blood*

The experiment described in the preceding section and diagrammed in Figure 4 was repeated utilizing a cyst suspension in inactive blood. A similarly prepared chamber without charcoal served as a control. Because of the sub-threshold titer of hormone in the culture medium, no development was expected and none was observed. Special attention was centered on the microscopic appearance of the cysts as a criterion of their viability. After seven days, the two rows of cultures furthest from the charcoal remained normal in all respects and could not be differentiated from the controls. By contrast, the cysts in the row nearest to the charcoal showed slight darkening and an abnormal degree of granularity. In the absence of appropriate tests, we cannot state with certainty whether these cultures remained viable. However, there could be little doubt that their abnormal characteristics were far less prominent than previously noted for equivalent cultures in the presence of hormone.

Discussion

The experimental results indicate that the development of spermatocytes requires the presence, not only of hormone, but also of a further developmental factor which the hormone-stimulated cells generate within the culture medium. This factor is soluble in blood and volatile. Consequently, it distributes itself between the culture medium and the surrounding gas phase. The amount of factor which a single culture can generate is limited, but can apparently be enhanced by increasing the volume of blood medium available per cyst. This suggests that the cysts generate the volatile factor from a non-volatile precursor within the medium and that it is this precursor which is limiting.

Though hormonally stimulated cells die in the absence of the volatile factor, among the population of cysts in any culture one observes a broad distribution of thresholds for stimulation by the factor. A single culture can equilibrate with the scanty gas volume of a sealed depression slide and still retain within itself a sufficient concentration of the factor to stimulate a high proportion of cysts. However, in larger chambers having an internal volume of 17 ml., eight cultures are required to establish and maintain an equivalent concentration. Hence, it may be calculated that a single culture can equilibrate with a gas volume of about 2 ml. before the concentration of the factor begins to limit the response of the cysts with high thresholds.

By virtue of the circumstances just described, individual cultures are protected against excessive loss of the volatile factor by the presence of other cultures which are likewise generating the factor. Consequently, the individual cultures interact *via* the gas phase which they share.

Though the chemical nature of the volatile factor is a matter for further investigation, the information already at hand permits certain inferences. The volatility, in itself, indicates that the factor is neither a protein nor any other very large molecule. Moreover, its failure to react with acid or alkali is suggestive of a neutral molecule. The absence of detectable solubility in aqueous solvents means that the factor is neither hydrophilic nor strongly polar. Its definite, though limited, solubility in mineral oil indicates the presence of apolar groups. Though insect blood contains about 85 per cent water, the properties of blood as a solvent are complex and poorly understood; consequently, little can be inferred from the high solubility of the factor in this medium.

It is a curious fact that the present phenomenon most closely parallels the action of ethylene in relation to the ripening of fruit. As recently reviewed by Biale (1950), there is now general agreement among plant physiologists that unripe fruits, under the over-all stimulation of auxin, generate within themselves catalytic amounts of ethylene; the latter is then essential for the biochemical events associated with ripening. A single apple is said to produce a total of up to one cc. of ethylene gas, while experimentally one finds ethylene to be effective in concentrations as low as a few parts per million.

On the distant chance that ethylene might likewise be involved in the "ripening" of the insect sex cells, the gas was tested in several low concentrations and found inactive as a substitute for the volatile factor. However, it is not unlikely that the techniques developed by botanists in collecting and identifying ethylene may prove helpful in further study of the present problem. The testes of the intact insect receive an intimate tracheal supply, and it is probable that the volatile factor escapes from the tracheal system during the period of adult development. Presumably this loss is steadily replaced by a transformation of the precursor present in the blood.

Though the developmental factors in insect spermatocytes, as in the fruit of plants, could scarcely have been detected had they not been volatile, it is our opinion that volatility is a property only incidentally associated with the molecules in question. Of far greater interest is the demonstration that the reaction between cells and hormones can generate molecules which are catalytically active and prerequisite for the biological end-result.

SUMMARY

1. The *in vitro* development of spermatocytes of the Cecropia silkworm is inhibited when individual cultures are placed in large chambers rather than in depression slides. The inhibition is relieved by increasing the number of cultures in each sealed chamber—a finding which demonstrates that the individual cultures interact.

2. The basis of this interaction was studied in detail. Evidence was obtained that the interaction occurs *via* the gas phase within the chamber by virtue of the production by the cultures of a developmental factor which is volatile.

3. The role of this volatile factor was studied in relation to *in vitro* spermatogenesis. Its production was detected only in cultures containing spermatocytes and hormone. These components along with the volatile factor were then found necessary for the developmental response.

4. By suitable tests it was possible to show that the volatile factor was neither carbon dioxide nor any other normal constituent of air.

5. The factor is definitely soluble in insect blood, slightly soluble in mineral oil, and relatively insoluble in water. On the basis of present evidence, it appears to be a neutral, apolar molecule.

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